IMMUNOHISTOCHEMICAL LOCALISATION OF GHRELIN IMMUNO-REACTIVE CELLS IN VITAL ORGANS OF A FRESHWATER TELEOST *Channa punctatus* (Bloch, 1793)

SYNOPSIS

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STATE OF ART–

In the living world nucleotide energy is like a fuel for executing diverse biological functions not only essential for the survival of the organisms but also for their perpetuation of life (Błaszczyk 2023). This nucleotide energy is neither being created nor being destroyed as per the first law of thermodynamics which is being followed by all the living organisms in this living world. Rather the chemical energy of the food is being transduced by the active metabolism of the living cells which is then used for performing the biological work. In higher multicellular organisms getting or obtaining the food, feeding, digesting the food, absorbing the digested food and its assimilation is dependent upon, regulated and co-ordinated by numerous brain-gut peptides (Manoharan *et al* 2022). Among these one of the candidate hormone is Ghrelin, which makes the organism feel hungry therefore is known as a "hunger hormone" or Orexigenic agent (Albarran-Zeckler *et al* 2011). Ghrelin is secreted by X/A like oxytinic cells in rats and P/D1 cells in human which are abundantly present in stomach and gastrointestinal tract. Ghrelin is the member of brain-gut peptide family, first identified by Kojima *et al* (1999) in rat and human stomach extracts using reverse pharmacology strategy (Peeters *et al* 2003). Since then, many researchers have identified and localized the ghrelin producing cells not only in the sections of the different portions of vertebrate gut but also in pancreas, lungs, adrenal cortex, brain and gonads by applying immunohistochemical techniques (Wierup *et al* 2002). The uniqueness of the distribution of the endocrine cells in the gut is that these are present amidst the non-endocrine cell population, thus showing diffuse and dispersive nature of distribution. The literature review studies reveal that ghrelin is multifunctional hormone imparting pleiotropic effects on different body functions and regulation (Bowers 2012 and Bukhari 2022). One major research evidence gives clue about its involvement in hypothalamic pituitary-gonadal axis (HPG axis). And this axis responsible for gonadal function such as spermatogenesis, follicular development and gamete maturation through LH and FSH secretion. Hence ghrelin role for reproductive pathway opens new doors for fish healthy reproductive approaches and enhancement for fish breeding (Das *et al* 2019). There is a strong and significant cryptic association between Ghrelin and LH, which attracts many researchers to work on it. The research studies carried on mammals and fishes show

contrasting results. In one of the work it is argued that Ghrelin-LH relation are species specific because in mammal it is antagonist but in fish study it has been found to have agonist relationship, that is Ghrelin stimulates LH release (Heif *et al* 2010, and Grey & Chang 2013). Most of the work is reported form mammalian models and few in the fish model. No work on ghrelin immunohistochemical localisation in the fresh water teleost *Channa punctatus* has been reported so far. Due to above reasons and to fill the lacuna or gap in the research area, the present comparative investigation is designed to identify the Ghrelin immuno-reactive cells in some vital organs of a fish *Channa punctatus*. The findings of the experiment will provide useful data which can be applied in further research in the field of nutritional design and breeding of this particular fish.

INTERNATIONAL STATUS

The remarkable revolution has been observed in the study of fish gut endocrinology after the discovery and application of immunohistochemical technique since its first discovery in 1930-1940s. A good number of international researchers have since then worked in the identification and localisation of different gut endocrine cells in different fish species. Through IHC studies scientists have also localised the presence and source of many of the gut hormones in brain and other tissues of the body, thus elucidating the brain gut cross talk and pleotropic roles of the gut hormones.

The first enlisted use of IHC was in the year 1942 by Coon *et al* who used fluorescently-linked antibody to visualize pneumococcal bacteria. Since then many international researches have been using IHC in the study of numerous different gut endocrine cells and their distribution in extra enteric sites in various vertebrate models.

Many international researchers have done remarkable work on Ghrelin IR cells localization on different parts or body on different animal model. Ghrelin is one of the recently first identified in rat and human extracts by Kojima *et al* (1999). Thereafter its occurrence is reported in mammals and Aves by Kaiya *et al* (2002) and Wada *et al* (2003), in Reptiles by Kaiya *et al* (2004) in Amphibians by Kaiya *et al* (2001) and Kaiya *et al* (2006), and in Fishes by Kaiya *et al* (2009) and Minura *et al* (2009).

Sakata *et al* (2002) and Wada *et al* (2003) have reported the presence of only closed type of ghrelin immuno-reactive cells in rat and chicken stomach. While in the stomach of rainbow trout, Sakata *et al* (2004) have shown the presence of both the morpho-types (open and closed) of ghrelin producing cells based on their immunohistochemical studies.

The immunohistochemical localization of ghrelin cells in gut and gut associated organs and extra gut regions such as brain, pituitary, thyroid, adrenal, kidney, placenta and gonads in various vertebrates have been worked out by many researchers abroad. Date *et al* (2000), Kamegai *et al* (2001), Gnanapavan *et al* (2002), Volante *et al* (2002), Gualillo *et al* (2003), Barreiro *et al* (2004), Lely *et al* (2004), Mondal *et al* (2005) and Zigman *et al* (2006). Some of the research studies are done on goldfish's brain and intestine by Sánchez-Bretaño *et al*, (2015) and Cabral *et al, (*2017). A few studies on ghrelin IR cells are found on rat's GI tract and brain samples by Cabral *et al* (2017), Li *et al* (2019), Perelló *et al (*2022).

In recent times many international researchers through immunohistochemical techniques are trying to explore the relation and role of Ghrelin in fish reproduction, by influencing the GnRH, FSH and Kluge *et al* (2013), Grey & Chang (2012), Sokołowska-Mikołajczyk (2009). Shepperd, Peng and Unniappan, (2012), Unniappan and Peter, (2005); Kaiya *et al*., (2008) Chen *et al*., (2009) Jönsson *et al*., (2010) Velasco *et al.* 2016, Volkoff *et al*., (2003) Park and Ahima, (2015), Levanti *et al.*, (2011) Lo Cascio *et al*., (2018) Garcia-Suarez *et al*., (2018) Hansen and Zeiske, (1998), Zhou, *et al* (2016), Ji, *et al* (2015), Zhong *et al* (2021), Hatef (2015), Mu~noz-Cueto *et al* (2020), Zhou *et al* (2010). Zhong *et al* (2021) in their review article have highlighted the role of ghrelin in fish reproduction. Relatively a very less researches are available on Indian teleost.

National Status –

Interestingly most of the Indian researchers have worked on gut physiology of teleost, in relation to its anatomy, histology and ultra-structural features. Mishra, *et al* (1991) reported about the functional peculiarities of the alimentary canal of *Lepidocephalicthys guntea.* Nagar and Khan (1957) revealed about the anatomy and histology of the alimentary canal of *Mastacembelus armatus* (Lacep).

Limited number of articles are available on IHC studies for localization gut endocrine cells in Indian teleost. The study of gut endocrine cells (Gastrin and Secretin secreting cells) through immunohistochemical method in Indian teleost, *Lepidocephalus guntea* and Indian Skipper frog, *Euphlyctis cyanophlyctis*, was initiated by Shukla and Rao (2012), and Rao and Shukla 2013) and Shukla *et al* (2015). Shukla and Rao (2020) have highlighted the role of Immunohistochemistry tool in exploring the endocrine physiology of gastro-intestinal tract of vertebrates. A majority of research work on fish neuro-endocrinology and

immunohistochemical localisation of various neuropeptides in fish brain has been contributed by Subhedar *et al.* 2012, 2014. Immunohistochemical localisation of NPY in brain, gut and gonads of mammalian models and freshwater cat fish species have been studied by some Indian authors Desai *et al* (2014), Ukil and Bidish, (2018) and Priyadarshini and Lal (2018).

So far Ghrelin IHC studies is concerned only Mondal *et al*, (2021) have worked on Ghrelin localisation in zebrafish's brain.

PROBLEMS TO BE INVESTIGATED –

The immunocytochemical study of Ghrelin secreting cells in gonad, intestine and brain of *Channa punctatus* to answer the following queries –

1) Whether the ghrelin immuno-reactive cells also localised in brain, gonads and other vital organs apart from the gut regions of *Channa punctatus?*
2) What is the difference in distribution and abundance in Ghrelin immuno-reactive cells in different organs of *Channa punctatus* during its feeding and non-feeding phases?
3) What is the difference in distribution and abundance in Ghrelin immuno-reactive cells in different organs of *Channa punctatus* during its breeding and non-breeding phases?
4) Is there any cross talk between gut, brain and gonadal axis through Ghrelin immuno-reactive studies in *Channa punctatus*?

OBJECTIVES OF INVESTIGATION AND MOTIVATION –

The main objectives are:

1) To identify the Ghrelin producing cells in the brain, gonads and alimentary canal of *Channa punctatus,* through immunohistochemistry.
2) To study the Ghrelin immuno-reactive cells during different phases of feeding and hunger.
3) To explore distribution of Ghrelin immuno-reactivity in during breeding phase of the fish.
4) To search the role of Ghrelin in gut-brain-gonadal axis of *Channa punctatus.*

HYPOTHESIS-

1. Ghrelin is a key hormone that regulated digestive physiology through its orexigenic effect.
2. Ghrelin is the hunger hormone increasing feeding urge and food intake, thus regulating the feeding behaviour of *Channa punctatus*.
3. Ghrelin also acts as novel crucial regulator of fish reproductive physiology and establishes link between nutritional status and reproduction in fish.
4. Ghrelin Cells are distributed throughout the different regions of gut, brain and gonads in *Channa punctatus.*

RESEARCH DESIGN AND METHODOLOGY

The primary data of the distribution and relative abundance of ghrelin secreting cells immunoreactivity will be generated through executing the IHC Protocol will be followed in the following manner:

Experimental steps.

1) The experimental fish model will be anesthetized by water soluble 2-Phenoxy ethanol.
2) The anesthetized fish will be dissected and subjected to trans cardinal perfusion to remove all the blood from the organism.
3) The injections of phosphate buffer saline with the suitable fixative will be given to the model (fish) into the lumen of the gut to allow it to pass out through the posterior opening of the tract to clear the gut content.
4) The desired section of the alimentary canal, brain and the gonad will be cut into small pieces and immerged in the same fixative for overnight.
5) Two sets of tissues will be taken, and treat it with two different ways, for one set the block will be prepared in paraffin wax and for the other set block will be prepared in CRYO ZT compound.
6) After getting two set of sections of the tissue, one will be done in normal microtome for paraffin block and another one in cryostat for frozen block.
7) Antigen Retrieval methods will be used for both the paraffin and cryo-sections

8) After all the above steps, the obtained sections will be treated for standard immunohistochemical methods to get the result and will be compared with control sections.

Year-wise plan of work and targets to be achieved:

FIRST YEAR:

1) Sections will be prepared of the brain, gonads and alimentary canal of different regions of *Channa punctatus*.
2) Standardization of the IHC protocol will be done because it is very crucial for any experiment depending upon the lab conditions.
3) After this we have to prepare the working dilution of primary antibody for dilution test.
4) Standard Immunohistochemistry methods for the detection of ghrelin endocrine cells will be applied to identify ghrelin producing cells in the different sections of different portions of the fish alimentary canal, gonads and brain tissues.
5) The number, size, shape, location etc., will be recorded during different phases of feeding and breeding.

SECOND YEAR: The above experiment will be repeated to confirm the result.

The tentative characterization for the current study are as follows:

1. Introduction: Concept of neuro-endocrine regulation of vertebrate gut and gonadal physiology.
2. Historical Resume of Immunohistochemistry and its application in the study hormone immuno-reactivity in vital organs of vertebrates.
3. Immunohistochemical localization of Ghrelin immuno-reactivity in fish gut, brain and gonadal tissues.
4. Materials and Methodology of IHC.
5. Results and Discussion
6. Conclusion.
7. Summary

TOOLS AND TECHNIQUES TO BE USED IN THE COLLECTION OF DATA

The research work of the proposed study will be carried out in the laboratory of Department of Zoology, St. Xavier's College, Ranchi, using instruments like cryostat and normal microtome to obtain the cryo-section as well as paraffin sections respectively. Standardization of IHC protocol will be done. Dilution test and Antigen Retrieval steps will be executed prior to running the Standardized IHC protocol on the different sections of gut, brain and gonadal sections.

The exprimental protocol will require glassware and different chemicals mainly the primary Polyclonal antibody against Ghrelin. Secondary Conjugated Antibody kit EXTRA 3 kit, Chromogen: ACE 101 kit etc.

SIGNIFICANCE OF THE STUDY –

The immuno-histochemical localisation of Ghrelin secreting cells in brain, gonad and gut sections of *Channa punctatus* is significant in following manner-

1) This will help to understand the neuro-endocrine regulation of digestive and reproductive physiology of the fish.
2) The results will help to better understand the pleiotropic actions of Ghrelin in gastric carnivorous fish.
3) This study will be useful in tracing the ontogeny of Ghrelin producing cells in anaminotes, if compared with the results of secondary data from other species of anaminotes as well as aminotes.
4) *Channa punctatus* not only edible value but also has pharmaceutical potential therefore its breeding and production is vital. This investigation will help in formulating fish feed and increasing fish breeding, productivity and propagation to promote economic sector and blue revolution.

RELEVANCE TO THE PRESENT DAY PROBLEM AND NEED OF THE SOCIETY-

According to recent scenario, after China, India is the world's second-largest aquaculture nation and the third-largest fisheries producer. Blue Revolution showed the relevance of fisheries and aquaculture industries. From the past few recent year, shifting from capture based to culture based fisheries promote and boost the Blue Economy.

Hence the present research work could be best approach to enhance fish reproductive health and promote breeding and contribute to enhance not only Indian economy but also will be helpful in resolving global food insecurity to some extent which is one of the prevailing societal problem

CONTRIBUTION TO THE EXISTING KNOWLEDGE –

The aim of any research activity is to explore new facts to enrich the pre-existing knowledge. This current research aims to focus on the immuno-localization of Ghrelin producing cells in some of the vital organs of the *Channa punctatus*. This study which will help to know the explore new roles of Ghrelin hormone in this particular fish, since already existing reports in different mammalian and fish models show contrasting results. The present study will assist in exploring the neuro-endocrine regulation of digestive and reproductive physiology in case of a lower anaminote member. Thus adding to the pre-exiting knowledge in the field fish neuro-endocrinology, gut biology and reproductive physiology.

FUTURE ASPECTS –

The knowledge about Ghrelin location and function in fish biology so far is very limited therefore a lot of future work can be done:

- Ghrelin is basically a hunger hormone but also known to influence fish reproductive physiology through GnRH, FSH and LH regulation. Experiments can be designed to explore this area for promoting both fish feeding behaviour and reproductive health at the same time to regulate fish breeding and production.
- Fish feeding and breeding is not only regulated by Ghrelin, in fact it is regulated by the interplay between more than two dozen different hormones. Therefore, the protocol of the current study can be utilised in studying the role of other hormones.
- The results generated by all these researches will be beneficial in the management of fish reproduction and production to fulfil the protein rich dietary supplements for human consumption thus contributing to resolve the problem of global food insecurity.

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